Structural basis of pre-existing immunity to the 2009 H1N1 pandemic influenza virus

Rui Xu[1*], Damian C. Ekiert[1*], Jens C. Krause[2], Rong Hai[3], James E. Crowe, Jr.[2], Ian A. Wilson[1,4†]

[1]Dept. of Molecular Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA
[2]Dept. of Pediatrics and of Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, TN 37232, USA
[3]Dept. of Microbiology, Mount Sinai School of Medicine, New York, NY 10029, USA
[4]Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

* These authors contributed equally to this work.
† To whom correspondence should be addressed. E-mail: wilson@scripps.edu

Supporting Online Material

Materials and Methods

Expression, purification, crystallization and structural determination of CA04 HA

The gene corresponding to the ectodomain of hemagglutinin (HA) from A/California/04/2009 (CA04) was inserted into a baculovirus expression vector modified from pAcGP67A (BD Biosciences Pharmingen) (*S1*) and expressed as described (*S2*). The construct contains an N-terminal signal sequence for secretion, HA1 residues 11-329, HA2 1-174 (H3 numbering), a thrombin cleavage site, a trimerization foldon sequence, and a His_6-tag for purification.

Supernatant from the suspension culture of insect Sf9 cells was batch purified using Ni-NTA resin (Qiagen). Fractions containing HA were dialyzed against 20mM Tris-HCl, 50mM NaCl, pH8, and digested with TPCK-treated trypsin (New England Biolab, 1:1000 ratio) at room temperature overnight. The trypsin digestion removed the foldon and His_6 tags and cleaved the HA0 into HA1 and HA2, although they remain covalently linked by a disulphide bond. Digested HA was further purified through ion-exchange and size-exclusion chromatography. Purified HA was concentrated to 10mg/ml in 20mM Tris-HCl, 50mM NaCl, pH 8.0 and crystallized by mixing with an equal volume (1μl each) of precipitant solution using the sitting drop, vapor diffusion method at 22.5°C. The precipitant in the reservoir contained 27% PEG-MME 2000, 0.1M Tris, pH 8.8.

Crystals were flash cooled in liquid nitrogen after brief soaking in reservoir solution plus 10% ethylene glycol. Datasets were collected at Stanford Synchrotron Radiation Lightsource (SSRL) beamline 9-2 and processed with HKL2000 (*S3*). The crystal structure of the CA04 HA was solved by molecular replacement with Phaser (*S4*) in the CCP4 package (*S5*) using a monomer of 1918 H1 HA (1RD8, A/South Carolina/1/1918) (*S1*). Six copies of HA monomers, in two trimer complexes, were located in the asymmetric unit. The initial model was automatically built in ARP/wARP (*S6*). The structure was then adjusted using COOT (*S7*) and refined with CNS (*S8*), REFMAC (*S9*) and BUSTER (*S10*). The final model contains 6 HA

monomers (2 trimers), with RMSDs between independent copies ranging from 0.34 to 0.59 Å. Residues 11-325 in HA1 and residues 1-171 in HA2 (H3 numbering) were built in all HA monomers. An N-terminal proline (Pro9) and glycine (Gly10) remaining from the secretion sequence, and a C-terminal serine (Ser175) from the thrombin cleavage site in the expression construct, were built in some monomers, but not in others, depending on the quality of the electron density. Electron density is observed at six potential N-glycosylation sites and a total of 14 carbohydrates residues were built in the model. Statistics for data collection and structure refinement are presented in table S1.

Expression, purification and crystallization of 2D1-SC1918 Complex

The A/South Carolina/1/1918 (SC1918) HA was expressed and purified as previously described (*S11*). Initially, 2D1 Fab was generated by proteolysis of intact IgG with papain and endoproteinase LysC. SC1918 complex crystals were obtained with 2D1 Fab generated by LysC digestion, but these failed to diffract to high resolution (presumably due to heterogeneous cleavage of the Fab by LysC). To overcome this obstacle, we resorted to recombinant expression of the 2D1 Fab in mammalian cells. Variable and constant mAb 2D1 lambda chains were cloned with end-to-end primers into a pEE12.4 vector (Lonza Group Ltd, Basel, Switzerland) with a modified mouse kappa leader using In-Fusion enzyme (Clontech, Mountain View, CA). The mAb 2D1 heavy variable chain was sequence-optimized and synthesized (GeneArt, Regensburg, Germany) and cloned into the pEE6.4 vector with a mouse kappa leader and a truncated heavy chain without affinity tags (Lonza Group Ltd). The plasmids were transformed into DH5alpha for EndoFree Plasmid Maxi preparation (Qiagen, Hilden, Germany). The DNA was transiently co-transfected into HEK 293F cells (Invitrogen, Carlsbad, CA) using PolyFect reagent (Qiagen) in a WAVE bioreactor (GE Healthcare Life Sciences, Piscataway, NJ). The supernatant was harvested on day 7 and purified through a gravity column with CaptureSelect Fab lambda resin (BAC B.V., GP Naarden, The Netherlands) in D-PBS and concentrated with 15 mL centrifugal filter units with 30 kD molecular weight cut-off (Millipore, Billerica, MA). The Fab was further purified by cation exchange chromatography (MonoS, GE Healthcare, using a gradient of 0.01-1M sodium acetate buffer, pH 5.0), and buffer exchanged into 10mM Tris pH 8.0, 50mM NaCl.

An excess of purified, recombinant 2D1 Fab was mixed with SC1918 HA and incubated overnight at 4°C to allow complex formation. The complex was purified from excess Fab by gel filtration in 10mM Tris pH 8.0, 150mM NaCl, buffer exchanged into 10mM Tris pH 8.0, 50mM NaCl, and concentrated to 10mg/mL for crystallization.

2D1-SC1918 complex crystals were grown at 4°C in sitting drops by vapor diffusion against a reservoir containing 16% PEG1000 and 100mM PIPES pH 6.7. The crystals were cryoprotected by stepwise addition (4% each) of ethylene glycol to the well solution, up to a final concentration of 28%, then flash cooled in liquid nitrogen.

Diffraction data were collected on the NIH General Medicine and Cancer Institutes Collaborative Access Team (GM/CA-CAT) 23ID-B beamline at the Advanced Photon Source at the Argonne National Laboratory. Diffraction images were integrated in HKL2000 (*S3*) and scaled and merged in XPREP (Bruker). Initial phases were determined by molecular replacement using Phaser (*S4*). A protomer from 1RUZ (HA) (*S12*), the V_H/V_L domains from 1RZF, and the C_H1/C_L domains from 1AQK were used as search models (*S13, S14*). Refinement was carried out in REFMAC5 (*S9*) and Phenix (*S15*) (including simulated annealing), alternating with manual rebuilding and adjustment in COOT (*S7*). TLS refinement was employed using 1-4

groups per chain (selected using TLSMD (*S16*)). Positive density for N-linked glycosylation was observed at 4 of the 5 predicted sites on HA, and a total of 9 sugar residues were built. Kabat numbering of the Fabs was assigned by the AbNum web server (http://www.bioinf.org.uk/abs/abnum) (*S17*). However, due to an apparent 9 base-pair duplication (*S18*) leading to a three amino-acid insertion between Kabat positions 58 and 59 of 2D1, homologous residues in strand C'' were shifted out of register towards the N- and C-termini of the strand. In order to maintain consistent numbering for the C'' residues on the basis of their location in the structure, one inserted residue was accommodated at position 52A (the typical location for insertions and deletions) and the remaining two were inserted residues at positions 62A and 62B. Final refinement statistics are summarized in Table S1.

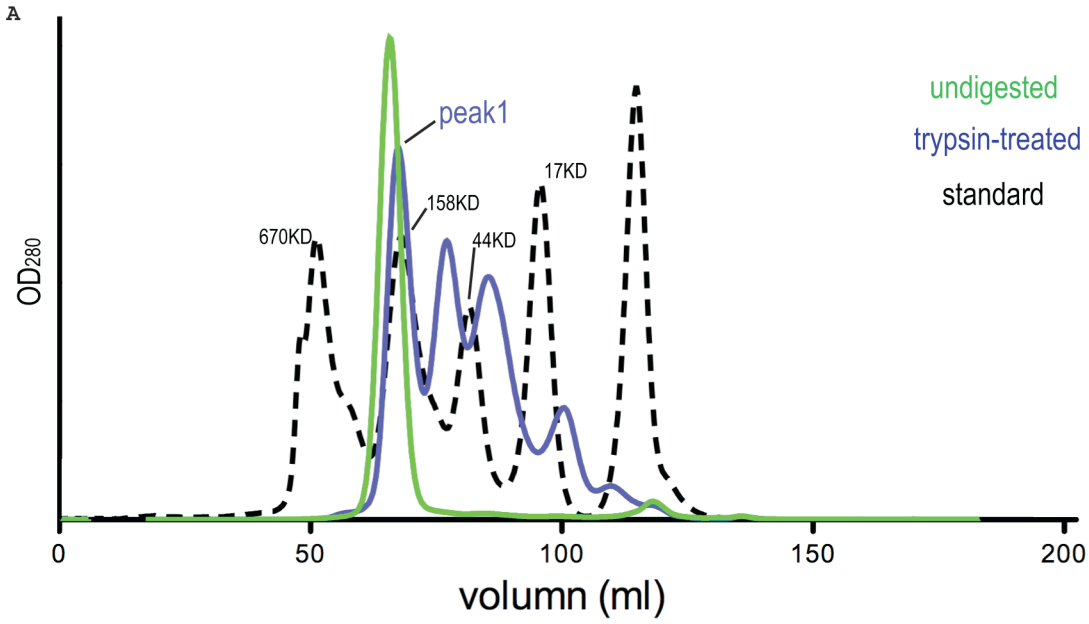
2D1 ELISA Against HA Panel

A panel of hemagglutinin proteins was expressed and purified essentially as described for SC1918 HA above (table S4). ELISA plates were coated with 50ng HA per well 50uL in Na-CO_3 buffer (pH 9.6) overnight at 4˚C. After blocking with 5% powdered milk in phosphate-buffered saline (pH 7.4) with 0.5% Tween-20, 50uL of 2D1 IgG at 10ug/mL in blocking buffer was added to the first well and serially diluted in 3-fold steps. The plate was incubated at room temperature for 2 hours, washed 5 times with blocking buffer, then incubated for an additional 2 hours at room temperature with a rabbit α-human, horseradish peroxidase-conjugated secondary antibody. After washing 5 times with phosphate-buffered saline (pH 7.4), the plate was developed with the TMB Substrate Kit (Pierce), the reactions were stopped by addition of 2M H_2SO_4, and the absorbance at 450nm was recorded for each well. Best-fit titration curves were calculated by non-linear regression to a sigmoidal function using the GraphPad package (Prism).

2D1 association with SC1918 mutants

Gene encoding influenza A/South Carolina/1/1918 HA protein was sequence-optimized for mammalian expression and synthesized by GenScript (Piscataway, NJ), based on the HA protein extracellular domain sequence (gi: 4325038), placed in-frame with a synthetic trimerization domain and a cleavable His tag for purification. The synthetic genes were cloned into a pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA). For mutagenesis of HAs, primers were designed using the QuikChange Primer Design Program for the QuikChange II XL kit (Stratagene, La Jolla, CA) and synthesized by Invitrogen. The DNA constructs were amplified in XL10 Gold bacteria (Stratagene) and purified with the EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany). HEK 293F cells (Invitrogen) were transfected with PolyFect reagent (Qiagen) and the resulting secreted recombinant HA protein was collected and purified on an AKTA FPLC using 5 mL HisTrap HP columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Affinity measurements were performed using full-length IgG molecules on an Octet QK interferometry instrument with anti-human IgG capture biosensors (FortéBio Inc, Menlo Park, CA). Antibody and antigen were diluted in 1X kinetics buffer with D-PBS. The data were analyzed with Octet software 4.0 and Origin 7.5 (OriginLab, Northampton, MA).

Figure S1. Purification of CA04. (**A**) Gel-filtration profile of CA04. CA04 expressed with a foldon tag eludes as a trimer on a Superdex 200 size-exclusion column (size 16/60) (profile in green). After trypsin digestion, a lower molecular weight trimer fraction (peak 1) and further degradation products could be separated (blue profile). Standard curves (black) were obtained by running a mixture of proteins with known molecular weights (marked on figure) under the same experimental conditions. (**B**) SDS-PAGE profile under reducing conditions of the trypsin-treated trimer fraction (peak 1) confirms removal of the foldon tag and cleavage of HA0 into HA1 and HA2.



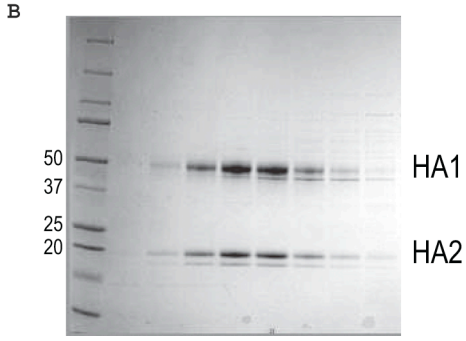


Figure S2. Comparison of 2D1 HCDR1 conformation with sequence-related Fabs. HCDR1 from 2D1 adopts an extended, open loop conformation when bound to HA. In contrast, other Fabs with sequences similar to 2D1 have a more compact configuration with the loop packing against the domain core. Depicted is the superposition of the V-gene derived regions (from the N-terminus up to the beginning of HCDR3) of 2D1 and the five most closely related Fabs from the PDB (based upon a BLAST search of the same region of 2D1). 2D1 is colored red, while the related structures are in grey, except for their HCDR1s that are highlighted as indicated below. For each, the percent conservation in HCDR1 (including surrounding residues), as well as the V-region overall, is given. 2D1 (PDB: 3LZF, red, 100% HCDR1, 100% overall), MEDI-493 (PDB: 2HWZ, green, 100% HCDR1, 85% overall), Yvo (PDB: 2AGJ, blue, 100% HCDR1, 83% overall), 12A11 (PDB: 3IFL, cyan, 100% HCDR1, 83% overall), 2F5 (PDB: 1U92, orange, 75% HCDR1, 79% overall), and PFA1 (PDB: 2R0Z, yellow, 92% HCDR1, 81% overall) (*S19, S20*).

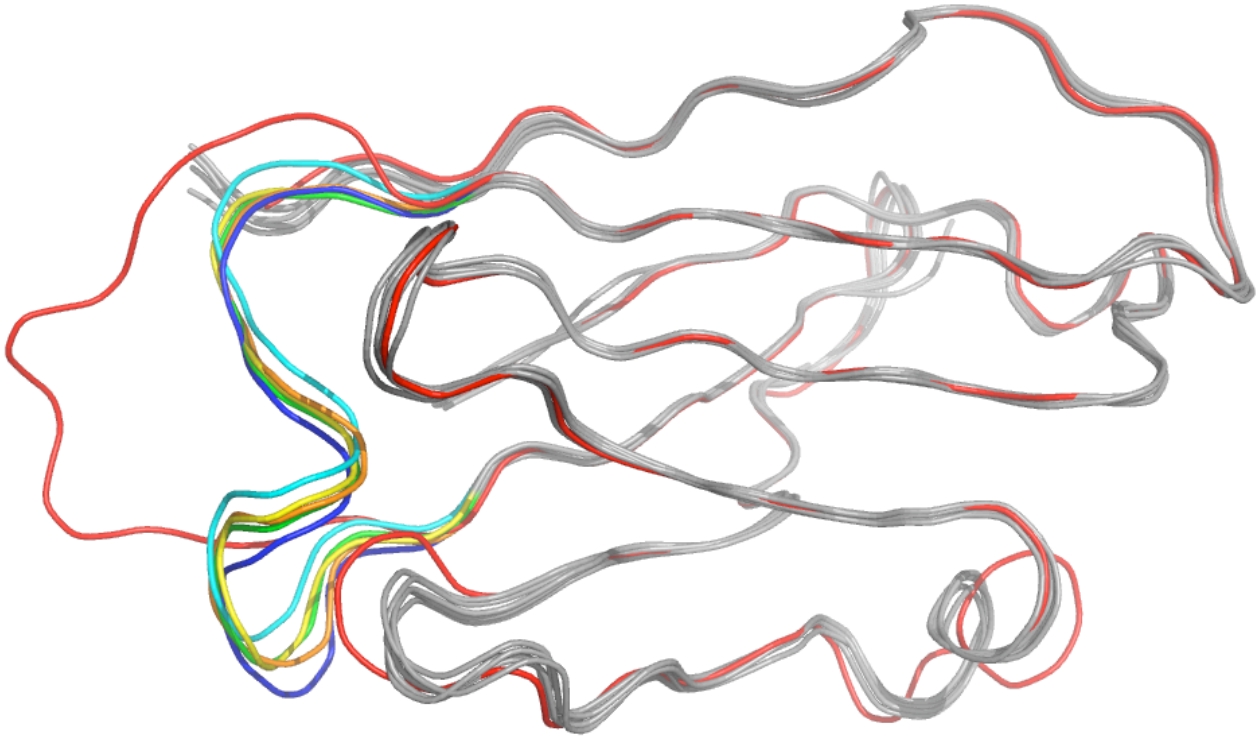


Table S1. Data collection and refinement statistics

Data collection	CA04	2D1-SC1918
Beamline	SSRL 9-2	APS GM/CA-CAT 23ID-B
Wavelength (Å)	1.03317	1.03333
Space group	P1	P321
Unit cell parameters (Å, °)	a = 67.7 , b = 118.3, c = 120.1 α = 117.4, β = 92.7, γ = 100.8	a = b = 161.7, c = 143.5 α = β = 90.0, γ = 120.0
Resolution (Å)	50 - 2.60 (2.69 – 2.60)[a]	50 - 2.80 (2.82 – 2.80) [a]
Observations	171,150	557,716
Unique reflections	93,210 (7,120) [a]	53,366 (639) [a]
Completeness (%)	91.0 (69.7) [a]	98.4 (61.5) [a]
$\langle I/\sigma_I \rangle$	8.7 (1.8) [a]	16.2 (1.8) [a]
R_{sym} [b]	0.08 (0.38) [a, b]	0.04 (0.50) [a, b]
Z_a [c]	6	1
Refinement statistics		
Resolution (Å)	45.0 - 2.60	49.7 - 2.80
Reflections (total)	90,481	50,122
Reflections (test)	4,548	2,596
R_{cryst} (%)[d]	19.0 [d]	23.0 [d]
R_{free} (%)[e]	25.0 [e]	25.9 [e]
Average B-value (Å^2)	66.3	82.3
Wilson B-value (Å^2)	61.7	90.4
Protein atoms	23,502	7,093
Carbohydrate atoms	184	120
Waters	443	0
RMSD from ideal geometry		
Bond length (Å)	0.009	0.016
Bond angles (°)	1.23	1.78
Ramachandran statistics (%)[f]		
Favored	93.4	93.7
Outliers	1.8	0.2
PDB ID	3LZG	3LZF

[a] Numbers in parentheses refer to the highest resolution shell.
[b] $R_{sym} = \Sigma_{hkl} | \langle I_i \rangle | / \Sigma_{hkl} I_i$, where I_i is the scaled intensity of the i^{th} measurement and $\langle I_i \rangle$ is the average intensity for that reflection.
[c] Z_a is the number of HA monomers per crystallographic asymmetric unit.
[d] $R_{cryst} = \Sigma_{hkl} | F_o - F_c | / \Sigma_{hkl} | F_o |$ x 100
[e] R_{free} was calculated as for R_{cryst}, but on a test set comprising 5% of the data excluded from refinement.
[f] Calculated using Molprobity (*S21*).

Table S2. Antigenic site variation in other H1 HA sequences compared to CA04. Sequences are retrieved from the National Center for Biotechnology Information (NCBI) Influenza Virus Resource. For seasonal H1 HAs from 1977 to 2009, only the vaccine strains are selected as representatives. The numbers of amino-acid sequence differences in each antigenic site from each H1 HA to CA04 are indicated, as well as % differences total (all antigenic sites) and in the Sa site. The numbers of potential glycosylation sites in Sa are also indicated. Human sequences are compared from 1918-1957 corresponding to evolution of H1N1 from the 1918 pandemic and then from 1977-2007 corresponding to reintroduction of the H1N1 in 1977 and to vaccine strains thereafter. Comparison with some representative swine flu H1 HAs from 1930-2007 is shown at the bottom of the Table.

Antigenic site (number of residues)	Sa (13)	Sb (12)	Ca1 (11)	Ca2 (8)	Cb (6)	Total (50)	Total (%)	Sa (%)	Number of glycosylation sites in Sa
A/South Carolina/1/1918	1	2	3	3	1	10	20	7.7	0
A/Wilson-Smith/1933	3	5	3	4	3	18	36	23.1	1
A/Puerto Rico/8/34	5	5	5	4	4	23	46	38.5	0
A/Phila/1935	5	5	3	3	3	19	38	38.5	1
A/Melbourne/35	4	5	3	3	2	17	34	30.8	1
A/Alaska/1935	5	5	5	4	4	23	46	38.5	0
A/Henry/1936	3	4	3	2	3	15	30	23.1	0
A/Hickox/1940	5	9	4	4	4	26	52	38.5	1
A/Bellamy/1942	7	6	3	4	4	24	48	53.8	2
A/Weiss/43	7	7	3	3	4	24	48	53.8	0
A/Iowa/43	7	6	4	4	4	25	50	53.8	1
A/AA/Marton/1943	7	6	4	4	4	25	50	53.8	2
A/AA/Huston/1945	7	6	4	4	4	25	50	53.8	2
A/Cameron/1946	6	9	4	4	4	27	54	46.2	1
A/Fort Monmouth/1/1947	5	8	4	4	4	25	50	38.5	1
A/Albany/4835/1948	6	8	4	5	4	27	54	46.2	3
A/Roma/1949	5	8	4	5	4	26	52	38.5	2
A/Fort Worth/50	7	9	5	3	4	28	56	53.8	1
A/Albany/4836/1950	6	8	4	5	4	27	54	46.2	2
A/Albany/12/1951	6	8	4	5	4	27	54	46.2	3
A/Albany/1618/1951	6	8	4	5	4	27	54	46.2	3
A/Albany/14/1951	6	8	4	5	4	27	54	46.2	3
A/Malaysia/54	7	8	4	5	4	28	56	53.8	1
A/Leninggrad/1954/1	5	9	4	5	4	27	54	38.5	3
A/Denver/57	6	9	5	3	4	27	54	46.2	3
A/USSR/90/77	5	9	4	5	4	27	54	38.5	3
A/Brazil/11/1978	5	9	4	5	4	27	54	38.5	3
A/Chile/1/1983	5	9	4	5	4	27	54	38.5	3
A/Singapore/6/1986	3	9	4	4	4	24	48	23.1	3
A/Bayern/7/1995	5	9	4	4	4	26	52	38.5	2
A/Beijing/262/1995	7	9	4	5	4	29	58	53.8	1
A/New Caledonia/20/99	5	8	4	4	4	25	50	38.5	2
A/Solomon Island/3/2006	5	8	4	4	4	25	50	38.5	2
A/Brisbane/59/2007	5	8	4	4	4	25	50	38.5	2
A/swine/Iowa/15/1930	1	1	3	3	2	10	20	7.7	0
A/New Jersey/1976	1	1	3	3	2	10	20	7.7	0
A/swine/Ohio/511445/2007	2	0	2	2	0	6	12	15.4	0

Table S3. 2D1-SC1918 interactions and comparison to later human H1N1 HAs. Sa site residues in the H1 HA and K_Ds of mutants, where known, are highlighted in magenta, as in the figures, with additional contact residues outside the previously designated Sa site in black.

	2D1-SC1918/H1				SC1918 vs.			SC1918 Mutants	
	HA1 Residue	Fab Residue	Type (#) [a,b]	Dist. (Å) [a,b]	CA04 2009	PR8 1934	Bris59 2007	Mutation	K_D for 2D1 (mutant/WT)
1	125C	V_L91	H-Bond (1)	3.0					
2		V_L93	H-Bond (1)	2.7					
3		V_L91	VDW (2)						
4		V_L93	VDW (5)						
5		V_L95	VDW (1)						
6		V_L95A	VDW (3)						
7		V_L95B	VDW (2)						
8	126	V_L93	H-Bond (1)	3.2					
9		V_L93	VDW (5)						
10	128	V_H58	VDW (3)						
11		V_L91	VDW (9)						
12	129	V_H97	H-Bond (1)	3.2			N129[c]	N129K	9.0
13		V_H97	VDW (5)						
14		V_H100C	VDW (7)						
15	157	V_H52	SALT (1)	3.7					
16		V_H54	SALT (1)	3.0					
17		V_H52	VDW (1)						
18		V_H54	VDW (4)						
19		V_H56	VDW (1)						
20	158	V_H53	VDW (3)			G158E	G158N	G158E	5.4
								G158D	0.9
21	159	V_H31	VDW (3)		S159N	S159G	S159G	S159G	5.6
22		V_H99	VDW (1)					S159N	33.3
23	160	V_H97	H-Bond (1)	3.0			S160L	S160L	1.3
24		V_H99	H-Bond (1)	2.5					
25		V_H97	VDW (2)						
26		V_H98	VDW (2)						
27		V_H99	VDW (7)						
28	161	V_H99	H-Bond (1)	3.1					
29		V_H100	H-Bond (1)	3.1					
30		V_H99	VDW (2)						
31	162	V_H97	VDW (4)						
32		V_H100	VDW (1)						
33		V_H100A	VDW (3)						
34		V_H100C	VDW (2)						
35	163	V_H100	H-Bond (1)	2.7			K163N[d]	K163N[d]	9
36		V_H100	VDW (4)						
37		V_H100A	VDW (4)						
38		V_H100B	VDW (12)						
39	165	V_L32	VDW (2)			S165K		S165K	4.4
40	166	V_L93	SALT (1)	2.8		K166N		K166E	> 250
41		V_L31	H-Bond (1)	2.9				K166Q	> 250
42		V_L93	H-Bond (1)	2.7				K166P	> 250
43		V_L30	VDW (3)						
44		V_L31	VDW (3)						
45		V_L93	VDW (3)						
46	167	V_L30	H-Bond (1)	3.1					
47		V_L30	VDW (3)						
48	169	V_L29	VDW (1)		V169I		V169A		
49	197	V_H99	VDW (5)				N197T		
50		V_H100	VDW (2)						
51	246	V_H100B	H-Bond (1)	2.9					
52		V_H100B	VDW (1)						
53	248	V_H100	VDW (2)			T248N	T248N		

[a] Hydrogen bonding interactions were calculated with HBPLUS (*S22*). [b] van der Waals contacts and salt bridges were calculated using CONTACSYM (*S23*). [c] Mutation nearby at position 131 creates an NXT motif, likely leading to glycosylation of N129 in Brisbane2007 [d] Mutation adds a potential N-glycosylation site.

Table S4. HA isolates in panel tested for 2D1 binding

Short Name	Full Name	2D1 Binding
H1-SC1918	A/South Carolina/1/1918 (H1N1)	+
H1-CA04	A/California/04/2009 (H1N1)	+
H1-PR8	A/Puerto Rico/8/1934 (H1N1)	-
H2-Jap57	A/Japan/305/1957 (H2N2)	-
H3-HK68	A/Hong Kong/1/1968 (H3N2)	-
H3-Bris07	A/Brisbane/10/2007	-
H5-Viet04	A/Vietnam/1203/2004 (H5N1)	-
H7-Neth03	A/Netherlands/219/2003 (H7N7)	-

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